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Kinetics of the plastoquinone pool oxidation following illumination Oxygen incorporation into photosynthetic electron transport chain

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Abstract **The oxidation of the PQ-pool after illumination with 50 or 500 μmol quanta m^{-2} s^{-1} was measured in isolated thylakoids as the increase in ΔA_{263}, i.e., as the appearance of PQ. While it was not observed under anaerobic conditions, under aerobic conditions it was biphasic. The first faster phase constituted 26% or 44% of total reappearance of PQ, after weak or strong light respectively. The dependence on oxygen presence as well as the correlation with the rate of oxygen consumption led to conclusion that this phase represents the appearance of PQ from $PQ^{\cdot -}$ produced in the course of PQH_2 oxidation by superoxide accumulated in the light within the membrane.**

Keywords: Thylakoids; Electron transport chain; Plastoquinone; Oxygen reduction

1. Introduction

Plastoquinone pool (PQ-pool) oxidation estimated as an increase in the area over the chlorophyll fluorescence induction curve after illumination of thylakoids demonstrated biphasic kinetics in a seconds range with a first rapid phase followed by slow tail [1,2]. With the same approach, biphasic kinetics of PQ-pool oxidation were found also in leaf disks of *Arabidopsis* and it was stressed that its rate was insensitive to inhibitors of oxidases [3]. The reasons of such kinetics were not discussed. The components of PQ-pool are the only membrane-embedded electron carriers which are not included into the molecular assemblies where electron pathways are strictly determined. This circumstance provides them with more possibilities for reacting not only with adjoining carriers of photosynthetic electron transport chain (PETC). It was shown that PQ-pool oxidation did not occur in the absence of oxygen [4]. Oxygen concentration inside the thylakoid membrane can be estimated as 2–10 O_2 per 1000 Chl, with higher values for more hydrophobic areas where its solubility is higher. It is comparable with concentrations of the electron carriers of PETC. Oxygen was found to be reduced by PQ-pool when PQH_2 oxidation by cytb_6f-complex was inhibited [5]. According the data in [5] this reduction, occurs at first as the univalent reduction of dioxygen by $PQ^{\cdot -}$ followed by reduction of superoxide radical, $O_2^{\cdot -}$, by PQH_2. It is moderate and is saturated at low-light intensity [5], while the PQ-pool participation in oxygen reduction in the entire PETC, being low in weak light attains 70% in strong light [6]. To explain such dependence, it was hypothesized [6] that PQH_2 can reduce superoxide radicals produced in both PQ-pool and PS I.

In this work we have found that PQ-pool oxidation measured as PQ reappearance after switching off the light consisted of two phases; after stronger light the extent of the initial rapid phase was higher, that coincided with a higher rate of oxygen consumption in the light. The data are discussed as indicating the participation of superoxide generated within the membrane in the light, in the first phase of the post-illumination PQ-pool oxidation.

2. Materials and methods

Thylakoids from leaves of pea plants grown in a greenhouse were isolated as described [5]. The basic reaction medium contained 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 10 μM Gr D, 50 mM Hepes-KOH (pH 7.8); thylakoids were with 100, 15 and 30 μg Chl per ml in the measurements of absorbance changes, oxygen uptake, and Chl *a* fluorescence, respectively. Absorbance changes were measured, using a dual-wavelength spectrophotometer (Hitachi 553, Japan), in a quartz cuvette with optical length 5 mm and the suspension thickness 4 mm perpendicular to actinic light. The absorption changes at 263 nm, – the minimum in the reduced-minus-oxidized spectrum of PQ [7], – were corrected for changes in transmittance and/or redox-state of other components as follows. For every variant the kinetics of both $\Delta A_{243-283}$ and $\Delta A_{263-283}$ were recorded; the line connecting zero on the *Y*-axis at 283 nm ($\Delta A_{283-283}$) and the value of $\Delta A_{243-283}$ at 243 nm at any moment after switching off the light was taken as the base line; the difference between the point at 263 nm at this line and $\Delta A_{263-283}$ at a corresponding moment was taken as ΔA due to PQ appearance. The measurements started 2 s after cessation of illumination. The differential extinction coefficient at 263 nm was taken as 13 mM^{-1} cm^{-1} [8]. The anaerobic conditions were created by adding 10 mM glucose, 25 μ/ml glucose oxidase and 500 μ/ml catalase. Light-induced cyt *f* redox-changes were determined as $\Delta A_{554-545}$; the blue–green filter SZS-22 (Russia) screened the photomultiplier from scattered light. Chlorophyll *a* fluorescence was measured under stirring in square quartz cuvette using a PAM fluorometer (WALZ, Germany). Oxygen concentration changes in a stirred thylakoid suspension (3.2 ml) were measured at 21 °C in a glass vessel with a Clark-type oxygen electrode; in these experiments, sucrose was 0.1 M, and Gr D was

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, dinitrophenylether of 2-iodo-4-nitrothymol; Gr D, gramicidin D; MV, methyl viologen; PETC, photosynthetic electron transport chain; PQ, plastoquinone; PQH·, $PQ^{\cdot -}$ plastosemiquinone; PQH_2, plastohydroquinone; PS I, photosystem I; PS II, photosystem II; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine

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1 μM. The separate PS I operation was achieved by additions of 10 μM DCMU, 5 mM ascorbate and 0.1 mM TMPD to provide electron donation to plastocyanin/P700. A saturating amount of superoxide dismutase was added to reaction mixtures in these experiments to prevent the reaction of superoxide with ascorbate. It was also added in the experiments without donor pair to provide the same conditions in the both analyses. Light was filtered through a red cut-off ($\lambda > 600$ nm) and heat absorbing filters. Light intensity measured with a Li-Cor quantum meter was varied using neutral filters. Chlorophyll was determined in 95% ethanolic extracts [9]. Stock solutions of DCMU and Gr D were in dimethylsulfoxide.

3. Results

Under aerobic conditions, a gradual increase in absorption at 263 nm toward the initial dark level occurred after switching off the actinic light, and the fraction of PQ appearing was estimated as the ratio of the difference between 'aerobic' and 'anaerobic' ΔA_{263} to 'anaerobic' ΔA_{263} (Fig. 1A and A′). Under anaerobic conditions only a small and rapid (within 3–4 s) increase in absorption at 263 nm occurred, and the concentration of the reduced PQ molecules preserved after illumination with weak or strong light was similar, 22–24 per 1000 Chl (after correction for flattening effect at 263 nm), that is close to total PQ concentration in thylakoids [2,7]. The kinetics of PQ reappearance was biphasic, and the semi-logarithmic plots of the kinetics are shown as broken lines (Fig. 1A, A′ and inserts). The slopes of the fast phases after illumination with weak or strong lights were 0.08 s^{-1} and 0.14 s^{-1}, respectively, and the contributions of fast phase were 26% and 44%, when the possible slow phase contributions, which could be found by extension of a line approximating this phase to the intersection with *Y*-axis, were subtracted. The slopes of first quick phases could be considered as apparent 'constants' of the pseudo-first-order reactions characterizing PQ-pool oxidation in weak and strong light, respectively.

The extents of PQ-pool reduction under aerobic conditions under both light intensities were similar as it is seen from the chlorophyll *a* fluorescence yields (Fig. 1B and B′). Therefore a difference in the level of PQ-pool reduction was not responsible for the difference in the contributions of fast phase of PQ reappearance after illumination. The high-potential components of PETC might be the first acceptors of electrons from the PQ-pool, if they were oxidized in the light. Under our experimental conditions, we did not observe any light-induced redox changes of cyt *f* in both strong and weak light (Fig. 1C and C′) (in the presence of Mv and in the absence of Gr D it demonstrated the characteristic redox-changes, dashed line in Fig. 1C′). Thus, the initial rapid phase of PQH_2 oxidation could not be explained by electron flow toward FeS-Rieske centre, cyt *f* and plastocyanin.

Since we subtracted the small changes in absorption at 263 nm under anaerobic conditions, the oxygen has to be implicated into process of PQ-pool oxidation. The rate of oxygen consumption in the light did increase with the increase in light intensity under operation of whole PETC as well as under conditions where electron transfer occurred only through PS I. The oxygen reduction rates (Fig. 2) were calculated from oxygen consumption rates, using the stoichiometries: 4 e^- per 1 O_2 consumed, for the entire chain, and 2 e^- per 1 O_2 consumed, for 'isolated' PS I [10]. In both cases the rates were limited by oxygen reduction [6], and therefore it was possible to compare them. It is seen that electron transport increased more steeply in the entire PETC.

4. Discussion

The implication of oxygen in the process of PQ-oxidation allowed the comparison between the higher fast phase of PQ appearance in stronger light and the higher rate of oxygen reduction. A putative plastoquinone oxidase was not involved in the process determining the biphasic kinetics of PQ-pool oxidation in the above experiments. The addition of catalase after illumination of thylakoids increased the oxygen concentration in the thylakoid suspension to the level before illumination (not shown, but see [11]), and this indicated that the final product of oxygen reduction was H_2O_2, but not H_2O. The role of enzymes in the kinetics of PQH_2 oxidation was not detected in [3] (see Section 1).

During illumination there is some accumulation of superoxide within the thylakoid membrane, since $O_2^{\cdot-}$ is stable there over the seconds range [12,13]. Since superoxide production depended on the light intensity (Fig. 2, curve 1) superoxide accumulation apparently determined the extent of the fast phase of PQ appearance after switching off the light. The process could be the rapid production of $PQ^{\cdot-}$ in the reaction of PQH_2 with the accumulated $O_2^{\cdot-}$, followed by PQ appearance from both $PQ^{\cdot-}$ dismutation and $PQ^{\cdot-}$ oxidation. The difference in E_0' values of pairs $PQ^{\cdot-}/PQH_2$ (0.37 V) [14,15] and $O_2^{\cdot-}/H_2O_2$ (0.94 V) [16] provides high-equilibrium constant, $>1.5 \times 10^4$, of the reaction

$$PQH_2 + O_2^{\cdot-} = PQ^{\cdot-} + H_2O_2, \quad (1)$$

and the electrostatic repulsion does not hamper it, since pK_1 of PQH_2 is close to 11. An increase of this reaction in stronger light was confirmed by an increase of the intramembrane H_2O_2 production with increasing light intensity [11]. The produced $PQ^{\cdot-}$ could either dismutate,

$$2PQ^{\cdot-} + 2H^+ = PQH_2 + PQ, \quad (2)$$

or be oxidized by dioxygen

$$PQ^{\cdot-} + O_2 = PQ + O_2^{\cdot-}. \quad (3)$$

The difference of redox potentials of pairs $PQ/PQ^{\cdot-}$, −170 mV, and $O_2/O_2^{\cdot-}$, −160 mV, is not high. The dismutation is possibly more significant due to high-equilibrium constant, 10^{10} [17], however, it can be partially hampered by an electrostatic repulsion (pK of $PQ^{\cdot-}$ is 7.0) as well as the distance between $PQ^{\cdot-}$ molecules formed.

After exhaustion of the superoxide that was accumulated in the light, the production of additional (to that produced in the reverse reaction (2)) $PQ^{\cdot-}$ molecules sharply decreased. The oxidation of residual PQH_2 molecules could proceed autocatalytically as follows: reverse reaction (2) → reaction (3) → reaction (1) producing additional $PQ^{\cdot-}$, and so on. $PQ^{\cdot-}$ was produced in a limited amount, and this determined the slow second phase of PQ appearance in Fig. 1A and A′. Obviously, the same process determined the slow phase of the dark PQ-pool oxidation, which was measured using fluorescence induction [1–3]. The oxidation of reduced plastoquinone placed into egg yolk lyposomes is very slow: the second order rate constant is close to 10^{-7} μM^{-1} s^{-1} [18], and an apparent first-order rate constant we estimated from the data in this

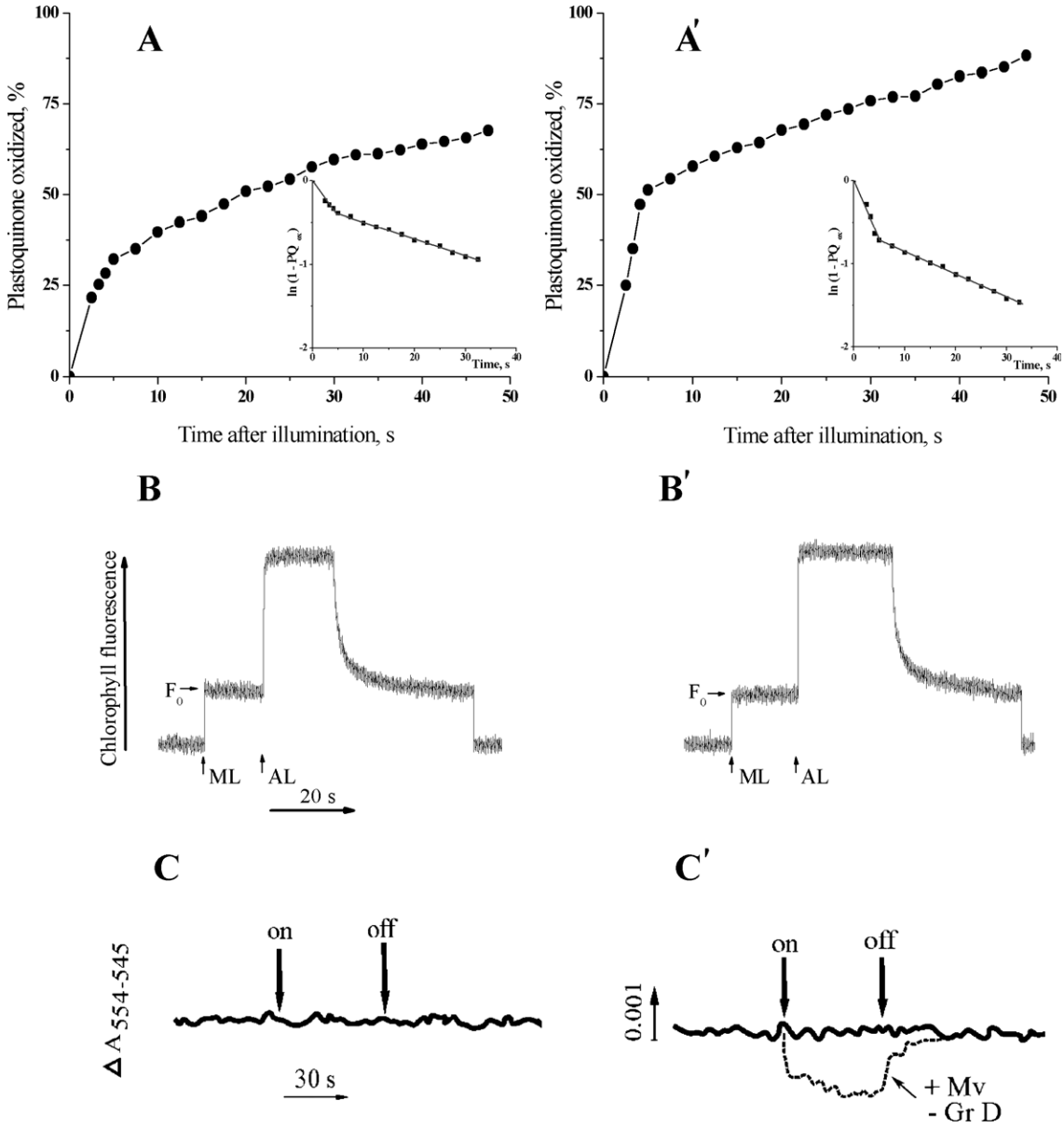

Fig. 1. The effects of light intensity on the PQ appearance after cessation of illumination (A, A′), chlorophyll *a* fluorescence yield (B, B′), light-induced redox-changes of cyt *f* (C, C′). In A, B, C and A′, B′, C′ the intensities of actinic light were 50 μmol quanta $m^{-2} s^{-1}$ and 500 μmol quanta $m^{-2} s^{-1}$, respectively.

work is 10^{-5}–10^{-4} s^{-1}. The apparent 'constants' determined from the initial slopes in Fig. 1A and A′ are considerably higher (see Section 3). The higher rate of PQ-pool oxidation in thylakoids than the rate of PQH_2 auto-oxidation in the liposomes was observed also earlier [4]. The difference may be just due to PQH_2 oxidation by the superoxide being produced within thylakoid membrane in the light.

The components of the acceptor side of PS I are recognized reductants of oxygen in thylakoids, and they were thought to be the main source of the superoxides within the membrane [16]; though the exact species which reduces dioxygen there were not specified. The superoxide can be produced also in PQ-pool according the reaction (3), however, the rate of this production cannot be high [5], and in both lights it should be similar, as this pool was reduced to the similar extent (Fig. 1B and B′). The ubisemiquinone formed at quinol oxidising site of mitochondrial and yeast bc_1-complexes is reductant of O_2 in the presence of inhibitors [19]. However, since the normal concerted oxidation of quinol by cytochrome complexes eliminates an appearance of long-lived semiquinone [20], and due to an absence of the experimental data about a participation of $PQ^{\cdot -}$ appearing at b_6f-complex in thylakoids, in dioxygen reduction, it is difficult to evaluate the contribution of the latter process in superoxide accumulation in the light under conditions of our experiments. However, we do not exclude it, and its possible dependence on light intensity.

An increase in the rate of oxygen reduction in PS I with increasing the light intensity (Fig. 2, curve 2) implied that stronger light resulted in a higher accumulation of superoxides within membrane owing to an increment of the superoxide

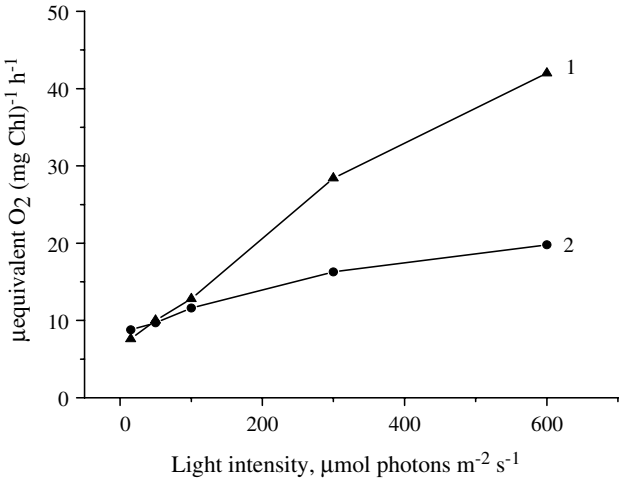

Fig. 2. The dependencies of the oxygen reduction rates in isolated thylakoids on light intensity. 1 – the entire electron transport chain, 2 – the Photosystem I operating separately (see Section 2).

production in PS I. The model (Fig. 3) proposed to explain the data presented now takes into account the previous data about an increase of PQ-pool participation in oxygen reduction with increasing the light intensity [6] as well as an increase of the intramembrane H_2O_2 production with increasing light intensity [11]. It also takes into account that plastoquinone molecules are distributed throughout the thylakoids [21]. The proposed microdomain membrane organization that can restrict PQ diffusion along the membrane [22] should not possibly influence the kinetics of PQ-pool oxidation since the average turnover time for rearranging domains was estimated as 60 ms, much less than the time range of the oxidation.

According to the model, the superoxide appearing in the light mostly in PS I oxidizes PQH_2 produced in PS II. This provides the direct connection between the photosystems. Moreover, this reaction produces $PQ^{\cdot -}$ molecules capable of

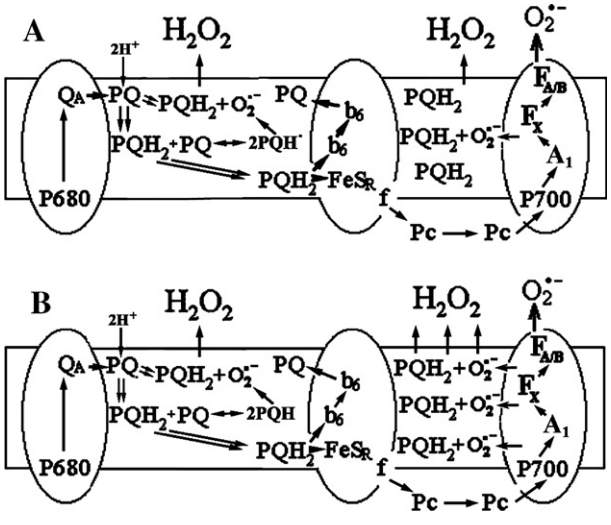

Fig. 3. A tentative scheme explaining the difference in the rates of PQ-pool oxidation after illumination with either weak (A) or strong (B) light as the result of oxidation of PQH_2 by superoxides accumulated to a greater extent in a stronger light owing to their production by membrane-embedded carriers of photosystem I.

being oxidized by O_2 (reaction (3)), and both reactions lead to an additional electron removal from the acceptor side of PS II; this can provide protection from photoinhibition when oxygen remains an electron acceptor, e.g., under limited CO_2 with closed stomata. The existence of these additional electron flows from PS II explains why the oxygen reduction in the entire PETC exceeds that in 'isolated' PS I in higher light (Fig. 2). The ratio PS II/PS I was repeatedly estimated to be higher than 1 [7,23]; and various mechanisms were proposed (see [24]) to 'diminish' this ratio to 1 in continuous light. Our scheme implies that, possibly, there is no necessity in such 'diminution'. The electron flow from 'PS II–PQH_2' to reduce superoxides inside the membrane can be also considered as needful to detoxify these reactive oxygen species here. The latter process was really observed [25].

The results of this and our previous work [5,6,11] give the possibility to speculate that oxygen is one of the electron carriers in PETC. Its incorporation into PETC is flexible, depending on light intensity (Fig. 3) as well as on other factors, which influence the electron flows from PETC to physiological acceptors.

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